IDENTIFICATION OF TUBERCLE BACILLI FROM INDIAN PATIENTS WITH PULMONARY TUBERCULOSIS

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Identification of Tubercle Bacilli from Indian Patients with Pulmonary Tuberculosis *,†

K. L. THOMAS, S. JOSEPH, T. V. SUBBAIAH & J. B. SELKON

Pretreatment cultures of bacilli from Indian patients with active pulmonary tuberculosis admitted to a controlled domiciliary chemotherapy study by the Tuberculosis Chemotherapy Centre, Madras, were subjected to a series of in vitro *tests designed to identify the bacilli as human or bovine tubercle bacilli, or as anonymous mycobacteria. For the purposes of comparison, pretreatment cultures from British patients with pulmonary tuberculosis were examined by the same series of identification tests.*

Cultures identifiable as mammalian tubercle bacilli were obtained from all the 341 Indian patients admitted to the chemotherapy study. Tests for niacin production were carried out on the cultures from 277 of these patients ; all gave positive results, indicating that the bacilli in question were Mycobacterium tuberculosis *var.* hominis. *The culture from the Indian patients yielded results similar to those of the cultures from the British patients in all the* in vitro *tests except the thiacetazone-sensitivity test. In this test the Indian cultures differed from the British cultures, being on the average less sensitive and showing greater variation in sensitivity among themselves.*

INTRODUCTION

A search of the literature has not revealed any previous reports of taxonomic studies of tubercle bacilli obtained from Indian patients. The objects of this paper are to report on the *in vitro* characteristics of cultures of tubercle bacilli obtained from Indian patients, to compare these characteristics with those of cultures from patients of British origin and to report on the anonymous mycobacteria encountered in this study. The cultures which are the subject of this paper were obtained from Indian patients participating in a controlled study of four different regimens of domiciliary chemotherapy (Tuberculosis Chemotherapy Centre, 1960) and from British patients attending chest clinics in England.

It was particularly important to identify whether the cultures obtained from Indian patients were *Mycobacterium tuberculosis* var. *hominis* or *Myco. tuberculosis* var. *bovis,* for anonymous mycobacteria can cause pulmonary disease, clinically and anatomically indistinguishable from tuberculosis (Timpe & Runyon, 1954; Wood, Buhler & Pollak, 1956 Nassau & Hamilton, 1957; Runyon, 1959). It has also been suggested (Palmer, 1953) that the high prevalence of low-grade tuberculin sensitivity encountered in some countries, including India (World Health Organization Tuberculosis Research Office, 1955), may be the result of infection with anonymous mycobacteria. Since Indian cultures of tubercle bacilli vary widely in their virulence in the guinea pig, many strains being attenuated (Frimodt-Møller, Mathew & Barton, 1956; Mitchison et al., 1960), guinea-pig inoculation is not a reliable identification test for Indian strains of tubercle bacilli. For the same reason animal virulence studies are not reliable as a means of differentiating between human and bovine strains of *Myco. tuberculosis* isolated from Indian patients. A series of *in vitro* tests was therefore selected which would differentiate mammalian strains of tubercle bacilli from the anonymous mycobacteria and identify strains of tubercle bacilli as either human or bovine.

* From the Tuberculosis Chemotherapy Centre, Madras, India. The Centre is under the joint auspices of the Indian Council of Medical Research: the Madras State Government, the World Health Organization and the Medical Research Council of Great Britain.

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METHODS

MYCOBACTERIAL CULTURES

Indian cultures

Cultures were obtained before treatment was started from each of the 341 patients, all of whom had active pulmonary tuberculosis, who were admitted to a controlled study of four different chemotherapeutic regimens (Tuberculosis Chemotherapy Centre, 1960). The patients admitted to this study *(a)* had sputum which either contained acid-alcohol-fast bacilli on smear examination (93.0% of the 341 patients) or yielded cultures that morphologically resembled *Myco. tuberculosis* (no patients failed to be admitted to the study because they yielded cultures of mycobacteria without this resemblance), *(b)* were aged 12 years or more, *(c)* were resident in Madras City and *(d)* had not, so far as was known, previously received more than two weeks of antituberculosis chemotherapy (95.3 % had received none). Three patients were, however, subsequently found to have had more than two weeks of antituberculosis chemotherapy before admission to the study.

It was the intention to examine by the series of identification tests, one pretreatment culture from each of the 341 patients. Since all the cultures could not be studied within six weeks of their becoming positive, a proportion (53 %) were stored at –20°C until examined. The pretreatment cultures from 54 of these 341 patients could not be examined by the series of identification tests because the culture selected for examination was either contaminated (33), failed to grow on subculture (12) or was mislaid (nine). Twenty-eight of the 33 cultures which were contaminated, and all the 12 cultures which failed to grow on subculture had been stored at –20°C. None of the contaminants was an anonymous mycobacterium. There thus remained cultures from 287 patients (including the three who had received more than two weeks of previous chemotherapy) which were studied by the series of identification tests. No culture was excluded from this investigation because of resistance to streptomycin, p-aminosalicylic acid (PAS) or isoniazid.

British cultures

Cultures were obtained from 77 newly diagnosed and previously untreated British patients with pulmonary tuberculosis attending a number of chest clinics in England. Specimens of sputum obtained from these patients were sent to the Unit for Research on Drug Sensitivity in Tuberculosis (Medical Research Council of Great Britain), Postgraduate Medical School of London, for culture. Positive cultures were dispatched by air to Madras. Cultures from British patients were selected for this study if they were sensitive to streptomycin, PAS and isoniazid. They were included in all the batches of *in vitro* identification tests set up, roughly in the proportion of one British to four Indian cultures.

SPUTUM CULTURE

The sputum from both the Indian and the British patients was cultured on Löwenstein-Jensen medium after treatment with 4% NaOH and washing with distilled water (Tuberculosis Chemotherapy Centre, 1959).

CONTROL STRAINS

Three control strains– *Myco. tuberculosis* strains H37Rv and BCG and the photochromogenic anonymous mycobacterium strain 0735 (Selkon & Mitchison, 1959)–were set up with each batch of tests. These strains were obtained from Dr D. A. Mitchison of the Postgraduate Medical School of London and were maintained by serial subculture on Löwenstein-Jensen medium.

MEDIA

The Löwenstein-Jensen medium referred to throughout the text did not contain potato starch (Jensen, 1955). The 7H-10 oleic-acid-albumin agar medium and 7H-10 Tween-albumin liquid medium were prepared as described by Cohn, Middlebrook & Russell (1959).

IDENTIFICATION TESTS

Cultures were examined for the following characteristics :

In vitro *tests*

Bacterial morphology. Bacterial morphology and acid-alcohol fastness were studied by examining, with a magnification of x 700, smears prepared from 4-week-old cultures grown on Löwenstein-Jensen medium and stained by the Ziehl-Neelsen

method (Mackie & McCartney, 1959). The organisms were regarded as typical mammalian tubercle bacilli if they were strongly acid-alcohol fast, slender rods without excessive beading or barring, averaging 2-6 μ in length and without branching.

Colonial morphology. Cultures incubated at 37°C for four weeks on Löwenstein-Jensen medium slopes and on 7H-10 oleic-acid-albumin agar plates were examined for colonial morphology, the former macroscopically and the latter with a plate microscope (magnification x 8.75). Colonies on Löwenstein-Jensen medium were regarded as typical tubercle bacilli if they had a moderate or coarsely granular mat surface and were buff-coloured. The typical characteristics of the colonies of tubercle bacilli on 7H-10 oleic-acid-albumin agar plates were a granular mat surface, an irregular edge, a grey periphery with a central umbo and heaping of growth at the edges of adjoining colonies.

Growth at 23°C. Two slopes of Löwenstein-Jensen medium were inoculated with a suspension containing either about 0.01 mg or about 1 mg (moist weight) of culture; one was incubated at 37°C and the other at 23°C. The slopes were examined for growth at four and five weeks. Cultures were regarded as typical of tubercle bacilli if they did not yield growth at 23°C.

Pigmentation. Two Löwenstein-Jensen medium slopes were inoculated and then incubated at 37°C, one exposed to the electric light of the incubator room and the other in a tightly closed light-proof box. After four weeks' incubation, the cultures were examined for pigmentation and then kept on the laboratory bench at room temperature (approximately 30°C) for two weeks, one culture being exposed to daylight and the other remaining in the dark. The degree of pigmentation was then re-examined. If the colonies were buff-coloured, and if no increase in pigmentation had occurred after exposure to daylight, the culture was reported as typical.

Catalase activity. Catalase activity was determined by a qualitative method (Tuberculosis Chemotherapy Centre, 1959). Cultures were tested for catalase activity after four weeks' incubation on Löwenstein-Jensen drug-free medium and on Löwenstein-Jensen medium containing 50 µg/ml isoniazid. In order to obtain growth on the medium containing 50 µg/ml isoniazid, the slopes were heavily inoculated with approximately 2 mg (moist weight) of the parent culture. Strains whose growth on the drug-free medium did not yield excessive catalase activity or whose growth on 50 µg/ml isoniazid had no catalase activity were regarded as typical tubercle bacilli.

Niacin test. The production of niacin was studied, using the method of Gilani & Selkon (1958). Two slopes of 7H-10 oleic-acid-albumin agar medium were heavily inoculated and incubated at 37°C. Growth on one slope was tested for niacin production after four weeks. If it was negative, the other slope was tested after six weeks' incubation. Cultures which gave positive results were regarded as *Myco. tuberculosis* var. *hominis* and those which were negative at the end of six weeks as either bovine or anonymous strains.

Cord formation. Tubercle bacilli, but not usually anonymous mycobacteria, have the ability to grow in tightly bound cords of parallel bacilli (Middlebrook, Dubos & Pierce, 1947; Selkon & Mitchison, 1959); this ability was examined in slide culture after 7-10 days' growth by the method of Sievers (1949).

Arylsulfatase activity. Arylsulfatase activity was examined in 2-week-old cultures in 7H-10 Tween-albumin liquid medium containing M/100 phenolphthalein disulfate by the method described by Whitehead, Wildy & Engback (1953). Human strains of tubercle bacilli do not possess detectable arylsulfatase activity (Whitehead, Wildy & Engback, 1953; Selkon & Mitchison, 1959).

Drug-sensitivity. The sensitivity of cultures to isoniazid and to p-acetamidobenzaldehyde thiosemicarbazone (thiacetazone) was determined by the method described by the East African/British Medical Research Council Thiacetazone/Diphenylthiourea Investigation (1960). For the thiacetazone-sensitivity tests, the slopes contained 0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 µg/ml thiacetazone and 1.0 % triethylene glycol (the solvent for thiacetazone). The results of the sensitivity tests have been reported as the minimal inhibitory concentration (MIC) of the drug or as the resistance ratio (RR)–namely, the MIC for the test strain divided by the MIC for the control strain, H37Rv.

In vivo *test*

Virulence in the guinea-pig. Cultures were tested for virulence in the guinea-pig by the method described by Mitchison et al. (1960). In brief, 1 mg (moist weight) of a 3-week-old subculture was inoculated intramuscularly into each of two guinea-

pigs, one of which was killed at six weeks and the other at 12 weeks. At post-mortem examination, the total extent of tuberculous disease in the spleen, liver, lungs and local glands was assessed as a score ranging from 0 to 100. The ratio of the score to the survival time in days was determined for each guinea-pig. The measure of virulence employed was the mean of the square roots of the ratios for the two guinea-pigs and has been termed the root-index of virulence (Mitchison et al., 1961). Root-indices of virulence of 0-0.59 were considered as indicating a low degree of virulence, those of 0.60-0.89 as indicating a moderate degree of virulence and those of 0.90 or more as indicating a high degree of virulence. Guinea-pigs were Mantoux-tested four weeks after infection, using 0.1 ml of 1 : 100 Old Tuberculin. The Mantoux tests were read after 48 hours and the results were expressed as the two diameters of the area of erythema taken at right angles to each other.

RESULTS

CULTURES EXAMINED BY THE SERIES OF IDENTIFICATION TESTS

The results obtained with the 287 Indian cultures that were examined by the series of *in vitro* identification tests will be presented in two sections, the first comparing the results obtained with 285 cultures which were regarded as tubercle bacilli with those obtained with the 77 British cultures of tubercle bacilli and the second dealing with the results of the two cultures which were regarded as anonymous mycobacteria.

The 285 Indian and 77 British cultures identified as tubercle bacilli

The results obtained with the series of identification tests on the 285 Indian cultures and the 77 British cultures regarded as tubercle bacilli are summarized in Table 1. The results of the tests for cord formation and arylsulfatase activity are not included in this table as these tests were performed on only a small proportion of the cultures at the start of this study. The great majority of the cultures were examined by all the tests, except the test for catalase activity of the growth on medium containing 50 µg/ml isoniazid and the test for growth at 23°C. Indeed, only 14 Indian and 12 British cultures were not examined by all the other tests. With the exception of one British culture (A420), which was not examined for colonial morphology on 7H-10 medium or for niacin production, none of the cultures missed more than one of the identification tests. The tests of colonial morphology and pigmentation were not done because of either contamination or poor growth of the culture. The niacin test was not performed on 14 cultures (eight Indian, six British) because they were examined at the beginning of the study, before this test was introduced.

The Indian cultures behaved similarly to the British cultures in all the tests and all the cultures yielded results typical of mammalian tubercle bacilli, with the exception of 12 cultures (eight Indian, four British) which yielded growth at 23°C. The results of the full series of identification tests for these 12 cultures are shown in Table 2. Seven of the eight Indian cultures and three of the four British cultures yielded growth of less than 20 colonies at 23°C. The remaining cultures (one Indian, one British) yielded between 20 and 100 colonies. None of the 12 cultures yielded results atypical of mammalian tubercle bacilli in any other test, none was resistant to isoniazid and the guinea-pigs infected with 11 of them were strongly Mantoux-positive (diameters of erythema, 15 mm x 15 mm). The root-indices of five of the eight Indian cultures were, however, indicative of moderate to low virulence in the guinea-pig, but this is in keeping with previous findings on the virulence in the guinea-pig of tubercle bacilli isolated from Indian patients (Mitchison et al., 1960). These 12 cultures have, therefore, been regarded as mammalian tubercle bacilli.

The 12 cultures of tubercle bacilli that yielded growth at 23°C were examined early in the study, when the test was performed by inoculating Löwenstein-Jensen medium slopes directly from a cultures, using a wire loop. This method inoculated approximately 1 mg (moist weight) of bacillary mass on to each slope. Eight of the nine Indian and four of the five British cultures tested by this method yielded growth at 23°C. Subsequently, the method was changed and the slopes were inoculated with a loopful of an aqueous suspension containing 4 mg

TABLE 1

RESULTS OF IDENTIFICATION TESTS ON INDIAN AND BRITISH PRETREATMENT CULTURES

Source of culture		Identification test									
		Bacterial morphology	Colonial morphology		Growth at:		Pigmentation		Catalase activity		Niacin test
			On L-J slope	On 7H-10 plate	37°C	23°C	In light	In dark	On drug-free slope	On 50 μg/ml isoniazid slope	
Indian patients	Number of cultures with results typical of *Myco. tuberculosis*	285	285	281	285	167	283	283	285	41	277
	Number of cultures with atypical results	0	0	0	0	8	O	0	0	0	0
	Number of cultures examined by the test [a]	285 100 %	285 100 %	281 98.6 %	285 100 %	175 61.4 %	283 99.2 %	283 99.3 %	285 100 %	41 14.4 %	277 97.2 %
British patients	Number of cultures with results typical of *Myco. tuberculosis*	77	77	70	77	25	77	77	69	17	71
	Number of cultures with atypical results	0	0	0	0	4	0	0	0	0	0
	Number of cultures examined by the test [b]	77 100 %	77 100 %	70 90.9 %	77 100 %	29 37.7 %	77 100 %	77 100 %	69 89.6 %	17 22.1 %	71 92.2 %

[a] The percentages shown are based on a total of 285 cultures.

[b] The percentages shown are based on a total of 77 cultures.

(moist weight) of bacilli per ml. None of the 163 Indian and 24 British cultures tested by this method of inoculating the slopes yielded growth at 23°C. The growth encountered at 23°C with the 12 cultures by the earlier method was thus presumably due to the very heavy inoculum used. The ability of tubercle bacilli to grow at 23°C when heavily inoculated has been reported elsewhere (Csillag, 1961).

In summary, all the 285 Indian and 77 British cultures had *in vitro* characteristics typical of mammalian tubercle bacilli. All the 277 Indian and 71 British cultures tested for niacin production were niacin-positive, and were therefore accepted as human strains.

Cultures identified as anonymous mycobacteuia

Two cultures (13986 and 15361) gave results which identified them as anonymous mycobacteria (Table 3). Culture 13986 yielded a deep-orange colour even on incubation in the dark and was thus a scotochromogen (Runyon, 1959–Group II). Culture 15361 yielded increased pigmentation only after exposure to light and was thus a photochromogen (Runyon, 1959–Group I). These two cultures have not been regarded as being the etiological agent of the patient's pulmonary disease for the following reasons. Culture 13986 was isoniazid-sensitive and not pigmented when first tested for its sensitivity to isoniazid. A further pretreatment culture from this patient was also sensitive to isoniazid and not abnormally pigmented. The isoniazid-resistance and abnormal pigmentation of culture 13986 only appeared on subculture after it had been stored for many months at –10°C. This culture was eventually shown to be a mixture of two different strains which were obtained in pure culture. One of these strains had the *in vitro* characteristics described in Table 3; the other had the characteristics typical of *Myco. tuberculosis* var. *hominis.* Culture 15361 was obtained from a patient whose other pretreatment culture was isoniazid-sensitive and not photochromogenic. From the first month of treatment and for more than 12 months, this patient consistently yielded isoniazid-resistant, catalase-

TABLE 2

RESULTS OF THE FULL SERIES OF IDENTIFICATION TESTS FOR THE EIGHT INDIAN AND FOUR BRITISH CULTURES WHICH YIELDED GROWTH AT 23°C

Source of culture	Culture No.	Identification test												
		Bacterial morphology	Colonial morphology		Degree of growth[a] at:		Pigmentation		Catalase activity	Niacin test	Cord formation	Arysulfatase activity	Sensitivity to isoniazid (MIC in μg/ml)	Guinea-pig test results	
			L-J slope	7H-10 slope	37°C	23°C	In light	In dark						Mantoux (1:100 OT) (mm)	Root-index of virulence
Indian patients	11385	T[b]	T	T	2+	1	T	T	2+	Not done	Positive	Negative	⩽0.2	18 × 20 17 × 19	0.53
	11514	T	T	T	2+	1+	T	T	2+	Not done	Positive	Negative	⩽0.2	22 × 26 17 × 24	0.62
	11731	T	T	T	3+	5	T	T	2+	Not done	Positive	Negative	⩽0.2	13 × 16 15 × 20	1.03
	13613	T	T	T	3+	3	T	T	2+	Positive	Positive	Negative	⩽0.2	17 × 19 18 × 18	0.55
	13996	T	T	T	3+	3	T	T	2+	Not done	Positive	Negative	GO.2	16 × 15 Not done	0.47
	14601	T	T	T	3+	16	T	T	2+	Positive	Positive	Negative	GO.2	Not done	0.96
	15129	T	T	T	3+	6	T	T	2+	Not done	Positive	Negative	⩽0.02	20 × 20 22 × 21	0.51
	15920	T	T	T	3+	4	T	T	2+	Not done	Positive	Negative	⩽0.2	20 × 15 21 × 17	1.07
British patients	A 420	T	T	C[c]	3+	6	T	T	2+	Not done	Positive	Negative	⩽0.2	16 × 20 20 × 17	1.15
	A 424	T	T	T	3+	8	T	T	2+	Not done	Positive	Negative	⩽0.2	17 × 17 16 × 22	1.52
	A 457	T	T	T	3+	2	T	T	2+	Not done	Positive	Negative	⩽0.2	18 × 18 21 × 20	1.33
	I 530	T	T	T	3+	1+	T	T	2+	Not done	Positive	Negative	⩽0.2	20 × 23 Not done	1.49

[a] Degree of growth: 3+= confluent growth; 2+= innumerable discrete colonies; 1+=100-20 colonies; figures below 20 without the plus sign = number of colonies.
[b] T = typical of mammalian tubercle bacilli.

TABLE 3
IDENTIFICATION TEST RESULTS FOR THE TWO CULTURES REGARDED AS ANONYMOUS MYCOBACTERIA

Identification test	Result			
	Culture No. 13986		Culture No. 15361	
Bacterial morphology :	Typical acid-alcohol-fast bacilli		Acid-alcohol-fast cocco- bacilli	
Colonial morphology :				
Löwenstein-Jensen medium	Smooth glistening surface, deep yellow in colour		Smooth mat surface, buff-coloured	
7H-10 medium	Dome-shaped, orange colonies with smooth glistening surface and entire edge		Fine granular, mat surface, grey colour, irregular edge	
Degree of growth [a] at:				
37°C	3+		3+	
23°C	2+		2+	
Pigmentation :				
In light	Orange		Light yellow	
In dark	Orange		Typical buff-coloured	
Catalase activity :				
On drug-free slope	2+		2+	
On 50 µg/ml isoniazid slope	1+		2+	
Niacin test:	Negative		Negative	
Drug sensitivity :	*First test*	*Second test*	*First test*	*Second test*
Streptomycin RR	1 [b]	1	16 [b]	16
Isoniazid MIC (µg/ml)	0.2 [b]	1	>50 [b]	>50
Thiacetazone MIC (µg/ml)	>32	>32	8	
Drug sensitivity of another pretreatment culture from the same patient:				
Streptomycin RR	0.5 [b]		0.5 [b]	
Isoniazid MIC (µg/ml)	0.2 [b]		0.2 [b]	

[a] For explanation, see Table 2.
[b] Tests set up when the culture was first isolated.

negative cultures which were not abnormally pigmented or photochromogenic. A culture obtained after 12 months of treatment was examined by the full series of identification tests and classified as *Myco. tuberculosis* var. *hominis.*

CULTURES NOT EXAMINED WITH THE SERIES OF IDENTIFICATION TESTS

Cultures obtained before the start of treatment from 54 of the 341 patients admitted to the chemotherapy study were not studied with the series of identification tests for the reasons given earlier (see page 748). Two cultures from each of 47 of these 54 patients and one culture from each of the remaining seven patients obtained before the start of treatment were, however, tested when first isolated for their sensitivity to isoniazid. After the sensitivity tests had been read, qualitative catalase tests were carried out on the growth on the drug-free slope and on any isoniazid-containing slopes which yielded growth of 20 or more colonies. The tubes were then left on the laboratory bench exposed to daylight at room temperature (approximately 30°C) for two to four days and re-examined for the development of abnormal pigmentation.

Of the 54 patients, 52 yielded cultures which were sensitive to isoniazid (MIC < 0.2 µg/ml). As none of the cultures from these 52 patients showed excessive catalase activity or was abnormally pig-

TABLE 4

SENSITIVITY OF INDIAN AND BRITISH CULTURES OF TUBERCLE BACILLI TO THIACETAZONE

Minimal inhibitory concentration (µg/ml)	Number of cultures								Resistance ratio	Number of cultures			
	20-colony MIC				100-colony MIC					20-colony RR			
	Indian cultures		British cultures		Indian cultures		British cultures			Indian cultures		British cultures	
	No.	%	No.	%	No.	%	No.	%		No.	%	No.	%
< 0.25	6	2.7	7	10.6	21	9.3	15	22.7	< 0.25	16	7.1	2	3.0
0.5	38	16.9	25	37.9	41	18.2	28	42.4	0.5	34	15.1	22	33.3
1	37	16.4	23	34.8	56	24.9	21	31.8	1	53	23.6	25	37.9
2	100	44.4	11	76.7	85	37.8	2	3.0	2	77	34.2	17	25.8
4	34	15.1	0	0.0	21	9.3	0	0.0	4	33	14.7	0	0.0
8	3	1.3	0	0.0	0	0.0	0	0.0	8	5	2.2	0	0.0
16	2	0.9	0	0.0	0	0.0	0	0.0	16	1	0.4	0	0.0
32	0	0.0	0	0.0	0	0.0	0	0.0	32	1	0.4	0	0.0
>32	5	2.2	0	0.0		0.4	0	0.0	>32	5	2.2	0	0.0
Total	225	99.9	66	100.0	225	99.9	66	99.9	Total	225	99.9	66	100.0

mented or photochromogenic, and all were sensitive to isoniazid, they were regarded as *Myco. tuberculosis.* (Confirmation was obtained for 17 of these 52 patients from cultures obtained during treatment, which were examined by the series of identification tests and classified as *Myco. tuberculosis* var. *hominis.)* One patient yielded one culture sensitive to isoniazid and another culture resistant to isoniazid (growth on 5 µg/ml, but not on 50 µg/ml). Neither of these cultures was abnormally pigmented and the growth of the isoniazid-resistant culture on the 5 µg/ml slope was catalase-negative. Both cultures were therefore probably tubercle bacilli. A further culture obtained from this patient after 12 months' treatment was examined by the full series of identification tests and identified as *Myco. tuberculosis* var. *hominis.* The remaining patient yielded two cultures which had a low degree of resistance to isoniazid (MIC of 1 µg/ml). One of these two cultures was tested for guinea-pig virulence and proved to be highly virulent (root-index of virulence, 1.12). It was therefore regarded as a culture of mammalian tubercle bacilli.

In summary, all the 54 patients yielded cultures which have been considered to be mammalian tubercle bacilli.

NOTES ON THE IDENTIFICATION TESTS

Catalase activity of growth on medium containing 50 µg/ml isoniazid

Only 40 of 188 Indian and 17 of 54 British cultures of tubercle bacilli yielded any growth on the 50 µg/ml slope, even though a heavy inoculum of approximately 2 mg (moist weight) of culture was used. The failure to obtain growth with such a large proportion of the cultures was thus a serious limitation of this test. It is possible, however, that a larger inoculum may prove more satisfactory in this respect.

Niacin production

Niacin production was tested for on 271 Indian and 67 British cultures of tubercle bacilli after four weeks' incubation at 37°C. Positive results were obtained with 263 (97.0 %) of the Indian cultures and 65 (97.0%) of the British cultures. The remaining eight Indian and two British cultures were, however, positive when tested after six weeks' incubation. The control strain BCG was tested in 49 of the 51 batches of niacin tests and strains H37Rv and 0735 in all 51 batches of tests. Strains BCG and 0735 were niacin-negative in all tests and strain H37Rv

TABLE 5

RESULTS OF REPEAT THIACETAZONE-SENSITIVITY TESTS ON CULTURES WITH RESISTANCE RATIOS OF 4 OR MORE IN THE FIRST TEST

	20-colony MIC at first test							20-colony RR at first test						
	4 µg/ml		8 µg/ml or more		Total			4		8 or more		Total		
Number of cultures	34		10		44			33		12		45		
Number of cultures retested	25		7		32			30		9		39		
20-colony MIC on retest (µg/ml)	No.	%	No.	%	No.	%	20-colony RR on retest	No.	%	No.	%	No.	%	
<1	5	20	1	(14)[a]	6	79	< 1	6	20	3	(33)	9	(23)	
2	12	48	2	(28)	14	44	2	9	30	2	(22)	11	28	
4	5	20	2	(28)	7	22	4	10	33	2	(22)	12	31	
8	1	4	1	(74)	2	6	8	3	10	1	(11)	4	10	
16	0	0	1	(14)	1	3	16	0	0	1	(11)	1	3	
32	1	4	0	(0)	1	3	32	0	0	0	(0)	0	0	
>32	1	4	0	(0)	1	3	>32	2	7	0	(0)	2	5	

[a] Percentages based on fewer than 25 observations are enclosed in parentheses, as an indication of the small totals.

was always niacin-positive. This method of testing for the presence of niacin can thus be relied on to differentiate human strains of tubercle bacilli from bovine strains and anonymous mycobacteria, provided that the cultures are incubated at 37°C for at least six weeks.

Thiacetazone-sensitivity test

The thiacetazone-sensitivity test proved unsatisfactory as an identification test for Indian cultures of tubercle bacilli, as these showed considerable variation in their sensitivity to this drug. In this respect, the Indian cultures differed from the British cultures. The distribution of the sensitivity to thiacetazone of 225 of the 285 Indian and 66 of the 77 British cultures identified as tubercle bacilli by the series of identification tests is set out in Table 4. Three distributions are presented: *(a)* the minimal inhibitory concentrations (MICs) inhibiting the growth of 20 or more colonies; *(b)* the MICs inhibiting the growth of 100 or more colonies; and *(c)* the resistance ratios (RRs) for the 20-colony end-point. With the 20-colony definition of growth, the mean MIC for the Indian cultures was between 1.0 and 2.0 µg/ml thiacetazone, whereas for the British cultures it was between 0.5 and 1.0 µg/ml thiacetazone. The Indian cultures also had a wider range of sensitivity to thiacetazone than the British cultures; the MIC for Indian cultures ranged from < 0.25 to >32 µg/ml thiacetazone (19.5% had MICs of >2 µg/ml), as compared with from < 0.25 to 2.0 µg/ml thiacetazone for the British cultures. The difference between the Indian and the British cultures was also shown by the 100-colony definition of growth, though the range of sensitivity of the Indian cultures to thiacetazone was somewhat reduced. The results of the sensitivity tests, when expressed as resistance ratios, were similar to those expressed as MICs.

In order to determine whether the Indian cultures, which on the 20-colony definition of growth yielded growth on 2.0 µg/ml thiacetazone or had RRs of 4 or more, really were resistant to these concentrations of thiacetazone, cultures which yielded such results were retested. The results of the repeat tests are shown in Table 5. Considering the results expressed as MICs, 12 (38 %) of the 32 cultures which grew on 2 µg/ml or more thiacetazone in the first test, and were retested, yielded growth on 2 µg/ml or more in the second test. This is an appreciably

higher proportion than the 20% of the 225 cultures which yielded growth on 2 µg/ml or more in the first test. The results expressed as RRs were similar; of the 39 cultures which had RRs of 4 or more in the first test and were retested, 19 (42 %) yielded similar results in the second test, as compared with 20% of the 225 cultures which yielded RRs of 4 or more in the first test. These findings suggest that with Indian cultures, although there was considerable technical variation in the test, there were also genuine differences between cultures in respect of their sensitivity to thiacetazone.

DISCUSSION

Of the 287 cultures of Indian patients that were studied by the full series of identification tests, 285 yielded cultures which were classified as mammalian tubercle bacilli. The remaining two cultures, which were classified as anonymous mycobacteria, were not regarded as the etiological agents of the patients' pulmonary disease, because *(a)* they were isolated from each patient on only one occasion and *(b)* both patients yielded other cultures, which were regarded as typical tubercle bacilli.

The 285 Indian cultures identified as mammalian tubercle bacilli behaved similarly to the British cultures in the series of in vitro identification tests, except in respect of their sensitivity to thiacetazone. The Indian cultures were on the average less sensitive to thiacetazone than the British cultures and also showed greater variation in their sensitivity. Part of the variation in thiacetazone sensitivity of the different Indian cultures was due to technical reasons, but part was due to genuine differences between the cultures from different Indian patients, a small proportion of cultures being resistant to 2 µg/ml thiacetazone. The difference between the Indian and the British cultures was demonstrated by both the 20-colony and 100-colony definitions of growth. It is of interest that a difference has previously been demonstrated between Indian and British cultures of tubercle bacilli in respect of their sensitivity to PAS (Selkon et al., 1960). In the case of PAS, however, the difference was present only when growth was defined as 20 or more colonies, and not when it was defined as 100 or more colonies.

All the 277 Indian cultures of tubercle bacilli tested for niacin production were positive and were therefore regarded as human strains. The absence of infection with bovine tubercle bacilli among our patients is a little surprising in view of the report that 1.8 % of the cattle and 2.8 % of the buffaloes in the Madras urban and adjacent rural areas are tuberculin reactions (Indian Council of Agricultural Research, personal communication). It is possible, however, that an appreciable proportion of the positive reactions was due to infections with mycobacteria other than the tubercle bacillus, or that transmission of infection to humans occurs infrequently in India because of the local custom of drinking milk only if boiled and the mild nature of the disease in Indian cattle (Mallick, Aggarwal & Dua, 1942; Iyer, 1944).

For 54 further patients, less complete information was available. Cultures from 53 of these patients were classified as tubercle bacilli on the findings that their growth was inhibited by 0.2 µg/ml isoniazid, that they were not photochromogenic and that they did not show abnormal pigmentation. Sensitivity to 0.2 µg/ml isoniazid is not, however, a completely reliable criterion on which to classify cultures as *Myco. tuberculosis,* for a small proportion of anonymous mycobacteria are inhibited by this concentration of isoniazid (Lester, Botkin & Colton, 1958 Marks & Trollope, 1960). The anonymous mycobacteria which have, however, been reported by other authors as sensitive to 0.2 µg/ml isoniazid were photochromogens (Lester, Botkin & Colton, 1958) and scotochromogens (Marks & Trollope, 1960) and not strains which showed pigmentation similar to tubercle bacilli (Runyon, 1959–Group III) which are invariably resistant to 0.2 µg/ml isoniazid (Jenkins, 1959; Marks & Trollope, 1960). Since the 53 cultures were not photochromogenic or abnormally pigmented, it seems reasonable, therefore to regard them as tubercle bacilli on the basis of their sensitivity to 0.2 µg/ml isoniazid. The culture from the remaining patient was isoniazid-resistant, but was regarded as *Myco. tuberculosis* because of its high degree of virulence in the guinea-pig (Wilson & Miles, 1955).

The failure to detect any cases of pulmonary disease simulating tuberculosis in which anonymous mycobacteria could be incriminated as the etiological agent suggests that the prevalence of infections due to these organisms may be lower in Madras City

than in some other areas–for example, Georgia, USA (Crow et al., 1957), Thinsdale, Ill., USA (Lester, Botkin & Colton, 1958) and Great Britain (Selkon & Mitchison, 1959). It must be emphasized, however, that the 341 patients studied in this report were a selected group. All the patients presented with symptoms and a high proportion had extensive disease. They were admitted to treatment only if they yielded at least one sputum specimen that was positive on smear or culture examination. Furthermore, the prevalence of low-grade tuberculin sensitivity may be lower in Madras City (Andrews et al., 1960) than in other areas in India (World Health Organization Tuberculosis Research Office, 1957).

SUMMARY

Cultures from 287 of 341 South Indian patients admitted to a controlled chemotherapy study were examined by a series of *in vitro* identification tests and compared with cultures from 77 British patients.

The cultures from 285 of the 287 Indian patients were identified as mammalian tubercle bacilli. Of the remaining two cultures, one was a mixture of a scotochromogenic anonymous mycobacterium and typical tubercle bacilli and the other was a photochromogenic anonymous mycobacterium. Neither of these two anonymous mycobacteria was regarded as the etiological agent of the patient's pulmonary disease because neither was isolated on more than one occasion and both patients yielded other cultures which were regarded as typical tubercle bacilli.

Cultures from the remaining 54 of the 341 patients were also regarded as mammalian tubercle bacilli but on the basis of a limited number of tests. Cultures regarded as tubercle bacilli were thus obtained from all the 341 Indian patients admitted to the chemotherapy study.

The cultures from 277 Indian patients were tested for niacin production; all yielded positive results and were therefore classified as *Myco. tuberculosis* var. *hominis*.

The Indian cultures of tubercle bacilli yielded results similar to those of the British cultures in all the *in vitro* identification tests except the test for sensitivity to thiacetazone. The Indian cultures differed from the British cultures in that they were on the average less sensitive and showed greater variation among themselves in their sensitivity to thiacetazone.

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RÉSUMÉ

Dans le cadre des études sur la tuberculose dans l'Inde, le Centre de Madras a mis à son programme l'identification des souches de bacilles tuberculeux isolées de malades de l'Inde, et leur comparaison avec des souches de malades en Grande-Bretagne. Il était utile, en vue des études de chimiothérapie, de déterminer si les malades de l'Inde étaient atteints de *Mycobacterium tuberculosis* var. *hominis* ou *bovis*, ou encore de mycobactéries indéterminées, capables de provoquer des affections pulmonaires qu'il est impossible de distinguer de la tuberculose. On a même attribué à ces dernières la sensibilité non spécifique à la tuberculine observée dans divers pays, l'Inde en particulier.

La comparaison portait sur les prélèvements de 28 malades de l'Inde et de 77 patients de Grande-Bretagne, effectuée par les méthodes in vitro. Les cultures d

285 des 287 patients de l'Inde furent reconnues comme appartenant à la variété *hominis* du bacille tuberculeux. Les deux autres étaient un mélange de bacille tuberculeux et de mycobactéries indéterminées, qui ne furent pas classées comme cause étiologique de la maladie, n'ayant été isolées qu'une fois des malades en question, et les deux malades présentant en outre des bacilles tuberculeux typiques.

La seule différence entre les souches de l'Inde et celles de Grande-Bretagne concernait la sensibilité à la thiacétazone. Les premières y étaient plus sensibles que les secondes (supportant 2 μg/ml) et présentaient une gamme de sensibilité plus étendue. Une différence analogue avait été notée, antérieurement, dans la sensibilité au PAS.

Le fait qu'aucune infection ressemblant à la tuberculose et due à des mycobactéries indéterminées n'a été décelée dans le groupe de malades de l'Inde, permet de penser que ce type d'infection est plus rare à Madras que dans les régions des Etats-Unis ou de Grande-Bretagne où des études ont été effectuées. Il faut noter cependant qu'il s'agissait, à Madras, d'un groupe de malades sélectionné, dont la plupart présentaient des lésions étendues. Ils n'étaient admis au traitement chimiothérapique que si la culture ou l'examen microscopique était positif pour la tuberculose. D'autre part, la sensibilité non spécifique à la tuberculine est moins fréquente à Madras que dans d'autres régions de l'Inde.

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